drous pyridine (3 ml) as described above. The mixture of products obtained after ether precipitation was first subjected to concentrated ammonium hydroxide treatment and then to pyridine–acetic acid–H_2O (5 ml, 3:14:1, v/v). The total mixture of products was then applied on the column of trityl–cellulose (2 × 10 cm) in 0.05 *M* TEAB containing 10% ethyl alcohol. The uv absorbing material eluted from the column contained 5% of impurities as judged by paper chromatography in solvent C. The product was completely digested with snake venom and had the constituent nucleotide ratio for d-pT:d-pA:d-pG of 1.96:0.96:0.93, respectively.

Nitrogen Isotope Effects on the Chymotrypsin-Catalyzed Hydrolysis of *N*-Acetyl-L-tryptophanamide

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Abstract: The hydrolysis of *N*-acetyl-L-tryptophanamide catalyzed by α-chymotrypsin at 25.0° is accompanied by a nitrogen isotope effect of 1.006 at pH 6.73, 1.010 at pH 8.00, and 1.006 at pH 9.43. The step in which the carbon–nitrogen bond is broken must be at least partially rate controlling in the enzymatic reaction. The variation of the isotope effect with pH requires that there be an intermediate in the acylation of the enzyme in addition to those which have been observed previously. The pH dependence of these reactions is more complex than has previously been believed.

A consistent and reasonable static picture of the structure of α-chymotrypsin has emerged from amino acid sequence studies[1–4] and X-ray structure studies,[5–10] but the dynamic picture of the active enzyme is much more poorly developed. Certain features of the dynamic structure of the enzyme are clear from mechanistic studies,[11–13] including the existence of an acyl enzyme, the existence of several important ionizations, the existence of more than one catalytically active conformation, and the existence of additional intermediates, but the precise mechanism of the enzymatic reaction remains unknown. Our present study delineates some additional features of this mechanism.

The existence of an acyl enzyme intermediate is now well established both for nonspecific[14–17] and for specific[12,18,19] substrates. The formation of this intermediate is rate determining for amide substrates, and its decomposition is rate determining for ester substrates.

The pH dependence of the activity of chymotrypsin indicates that two ionizable groups have primary control over the activity of the enzyme. The protonation of histidine-57, with a pK_a of about 7, causes loss of activity at low pH.[12,20–22] The ionization of the terminal amino group of isoleucine-16 at high pH induces a conformational change which results in the loss of activity. However, a more detailed study has revealed that the pH dependence of the activity of this enzyme is much more complex than this simple picture involving two ionizations. Thermodynamic studies of substrate and inhibitor binding[23,24] and careful studies of the temperature dependence of kinetic parameters[25,26] have shown that the enzyme can exist in any of several conformations, the exact state being governed by pH, temperature, and presence of substrates or inhibitors. Further, several of the conformations are catalytically active, and although there does not seem to be a large difference in rate constants among various forms, the enthalpies and entropies of activation for individual reaction steps differ among the various forms.

Rapid kinetic measurements on a number of substrates have been made.[27–30] Measurements of the

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rate of acylation of chymotrypsin by furylacryloyl-tryptophanamide[31] provide evidence for three intermediates: the noncovalent Michaelis complex, a second enzyme–substrate complex, and the acyl enzyme. The nature of this second complex is unknown. Its formation is sufficiently slow to be clearly visible on the stopped-flow time scale.

The existence of a tetrahedral intermediate in the acylation of chymotrypsin has been discussed by Caplow.[32] Comparison of substituent effects in acylation of chymotrypsin by ester and amide substrates suggests that the rate-determining step is different in the two cases. Formation of the tetrahedral intermediate is presumed to be rate determining for esters, and decomposition of the intermediate is rate determining for amides. Analogous intermediates have been implicated in the nonenzymatic hydrolysis of esters, amides, and other carbonyl derivatives,[33–37] and although some of the earlier evidence for such intermediates has recently been questioned,[38] the existence of such tetrahedral intermediates is an established part of mechanistic organic chemistry.

Thus the dynamic picture of the action of chymotrypsin continues to grow in complexity. Unfortunately, it is frequently not possible to identify the particular structural change associated with a particular rate process. We do not know, for example, whether the new intermediate discovered in the work of Hess, *et al.*, is the tetrahedral intermediate discussed by Caplow. Although it is generally assumed that the rate-limiting step in the chymotrypsin-catalyzed hydrolysis of amides is the step in which the carbon–nitrogen bond is broken, no direct evidence for this has been available until now.

The use of heavy-atom isotope effects in enzyme reactions has received only limited attention.[39,40] In our own work[41–44] we have shown that such isotope effects are useful in mechanistic studies in cases where a bond to the isotopic atom is being cleaved in the rate-determining step. Although the interpretation of the observed isotope effect is complicated by the multistep nature of these reactions, it is possible to interpret such isotope effects quite easily if adequate ancillary information is available. In a preliminary communication[45] we reported that the hydrolysis of *N*-acetyl-L-tryptophanamide is accompanied by a nitrogen isotope effect (k^{14}/k^{15}) of 1.010 at pH 8.0, 25°. We now report details of those measurements, together with measurements of the isotope effect at other pH values, and a comparison with the model studies of Harbison.[46] These results substantiate our earlier conclusion that carbon–nitrogen bond breaking is the rate-determining step in this reaction. They also provide evidence for the existence of another reaction intermediate in addition to those which have already been reported.

Results

Nitrogen isotope effects on the hydrolysis of *N*-acetyl-L-tryptophanamide catalyzed by α-chymotrypsin have been measured by comparing the isotopic compositions of two samples of NH_3: a sample isolated after hydrolysis of 5–10% of the amide present, and a sample isolated from a completely hydrolyzed amide sample. The ammonia samples were isolated, purified, and oxidized to nitrogen with hypobromite. Isotope effects can be calculated directly from these two isotopic compositions, with a small correction for per cent reaction in the low conversion sample.[42]

The results of measurements of the nitrogen isotope effect on the chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophanamide are summarized in Table I.

Table I. Nitrogen Isotope Effects on the Hydrolysis of *N*-Acetyl-L-tryptophanamide Catalyzed by α-Chymotrypsin at 25°

pH	% reaction	Isotope ratios[a] × 10⁶ Low conversion	100% conversion	k^{14}/k^{15}
6.73	9.9	9331	9381	1.0060
6.73	9.9	9331	9380	1.0055
6.73	9.9	9323	9379	1.0064
6.73	10.0	9321	9380	1.0067
6.73	9.6	9326	9383	1.0064
			Mean	1.0062 ±0.0004
8.00	11.9	9261	9333	1.0083
8.00	10.5	9274	9357	1.0091
8.00	10.0	9244	9349	1.0119
8.00	9.3	9257	9355	1.0111
8.00	9.6	9241	9335	1.0106
8.00	9.6	9265	9349	1.0095
			Mean	1.0100 ±0.0010
9.43	10.0	9324	9387	1.0071
9.43	10.0	9318	9371	1.0059
9.43	10.6	9320	9378	1.0066
9.43	9.7	9325	9376	1.0057
9.43	10.0	9323	9384	1.0069
			Mean	1.0064 ±0.0006

[a] Decade settings for the ratio m/e 29/28, corrected to tank standard = 9200.

The isotope ratios reported are decade settings on the isotope ratio mass spectrometer which have been corrected to a constant value of the nitrogen tank standard. These decade settings are proportional to isotope abundances and can be used directly in the calculation of isotope effects. The reproducibility of the entire

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procedure has been thoroughly checked. The absence of extraneous ammonia in the samples was confirmed repeatedly. The reproducibility of individual measurements made on samples prepared from the same batch of substrate is high, again confirming the validity of our procedure.

No reversion of the acyl enzyme to starting materials by reaction with ammonia occurred under the conditions of this study. A sample of 0.01 *M* *N*-acetyl-L-tryptophanamide was hydrolyzed with chymotrypsin at pH 9.43 in the presence of 0.002 *M* $^{15}NH_4Cl$. The reaction was stopped after 50% hydrolysis, the substrate was isolated and hydrolyzed, and the resultant ammonia was purified and oxidized to N_2, which was analyzed by mass spectrometry. No excess ^{15}N was found in this material, so no more than 0.01% of the acyl enzyme can react with ammonia and revert to starting material.

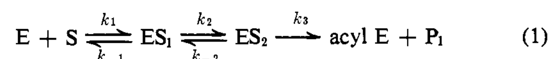
Discussion

In this study we report two observations which must be rationalized in any detailed explanation of the mechanism of action of chymotrypsin. (1) An appreciable nitrogen isotope effect is observed in the chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophanamide. (2) The magnitude of the isotope effect varies with pH. We will first discuss the implications of the magnitude of the isotope effect for the mechanism of action of chymotrypsin. We will then consider the variation of the isotope effect with pH and will show that the presently accepted mechanism is inadequate to explain this variation. Finally, we will derive a more general kinetic mechanism which is adequate to explain all that is known about the acylation of chymotrypsin by amide substrates.

The nitrogen isotope effect on the chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophanamide is in the range 1.006–1.010, the exact magnitude of the isotope effect depending on the pH. These isotope effects are similar in magnitude to those observed by Harbison[46] for the reactions of hydroxide ion with benzamide (1.004) and with phenylacetamide (1.006). The reaction of benzamide with hydroxide is accompanied by a substantial amount of oxygen exchange with water,[37] and clearly the mechanism involves rate-determining decomposition of a tetrahedral intermediate. Although the existence of a tetrahedral intermediate has not been clearly established in the case of chymotrypsin-catalyzed reactions, the similarity of the isotope effects in the enzyme reaction and in the hydroxide reaction suggests that the two have similar transition states.

An important difference between enzymatic reactions and the corresponding model reactions is the number of intermediates involved. The reaction of benzamide with hydroxide ion presumably involves only one intermediate—the tetrahedral intermediate—whereas the reaction of chymotrypsin with amides involves a number of intermediates[31]—the noncovalent Michaelis complex, a rapidly formed intermediate whose nature is unknown, possibly a tetrahedral intermediate, and probably additional intermediates.[47] One possible effect of the number of intermediates involved in the enzymatic reaction is that the observed isotope effect may be considerably smaller than the isotope effect on the bond-breaking step because other steps in the sequence occur at rates not too different from the rate of the bond-breaking step.

The work of Hess, *et al.*,[31] has shown that the acylation of chymotrypsin by amide substrates involves at least two intermediates, and thus can be expressed by eq 1. We have previously shown[43] that for this mechanism



$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_2 \xrightarrow{k_3} \text{acyl E} + P_1 \quad (1)$$

the relationship between the observed isotope effect and the isotope effect on the bond-breaking step is given by eq 2, where $R_1 = k_2/k_{-1}$ and $R_2 = k_3/k_{-2}$.

$$k^{14}/k^{15}(\text{obsd}) = \frac{k_3^{14}/k_3^{15} + R_2 + R_1R_2}{1 + R_2 + R_1R_2} \quad (2)$$

We have assumed that there is no isotope effect on any step except k_3 and that the reverse reaction (k_{-3}) does not occur.[48]

It is clear from eq 2 that the magnitude of the observed isotope effect cannot exceed the magnitude of the isotope effect on k_3. Considering the magnitudes of the isotope effects observed in the enzymatic reaction and in the model reaction, it seems likely that R_1 and R_2 in eq 2 are small compared to unity. In addition, R_1 and R_2 have been measured for furylacryloyltryptophanamide,[31] a substrate very similar to *N*-acetyl-L-tryptophanamide. It is likely that the values of R_1 and R_2 are similar in the two cases because the *R* values are ratios of rates for two reactions of one intermediate, rather than actual rate constants. In addition, our argument would not be changed even if R_1 and R_2 were tenfold or more different between the two substrates.

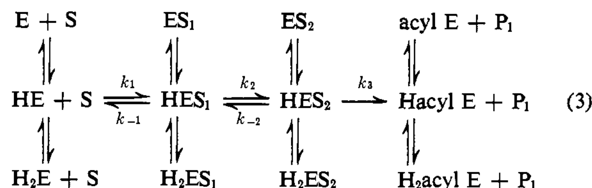
The values of R_1 and R_2 which are derived from the studies of furylacryloyltryptophanamide are both approximately 0.001. Thus, the additional steps in the kinetic scheme given by Hess, *et al.*,[31] do not influence the isotope effect and the observed isotope effect would seem to be equal to k_3^{14}/k_3^{15}. However, we will shortly show that the situation is not this simple.

pH Dependence. According to eq 2 the nitrogen isotope effect on a multistep enzymatic reaction depends on two factors: the isotope effect on the bond-breaking step (k_3^{14}/k_3^{15} in eq 2) and the partitioning ratios for intermediates prior to the bond-breaking step (R_1 and R_2 in eq 2). Variation in the observed isotope effect with pH might be due to variation in either of these factors or to some combination. We will consider the variation in isotope effect with pH in terms of this picture.

Equation 1 does not adequately represent the mechanism of the enzymatic reaction because it neglects the ionizations which the enzyme is known to undergo. Expansion of eq 1 to eq 3 is consistent with the results of steady-state kinetics. The observed isotope effect for eq 3 is still given by eq 2, even though ionizations

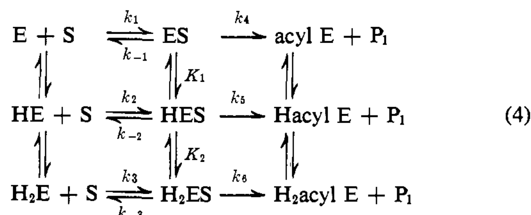
(47) We are considering here only the mechanism of acylation of chymotrypsin and not the decomposition of the acyl enzyme. We have shown that the acylation is irreversible under the conditions of our study, so our nitrogen isotope effects provide information only about the acylation steps.

(48) The assumption that there is no isotope effect on any step except k_3 in eq 1 is probably not precisely correct. However, it is certainly true that the isotope effect on k_3, which is the step in which the carbon–nitrogen bond is broken, is considerably larger than the isotope effect on any other step.



of intermediates HES_1 and HES_2 may affect the observed reaction rate. The important point is that the *fate* of the group of intermediates ES_1, HES_1, and H_2ES_1 (that is, the ratio of the rate of formation of HES_2 to the rate of return to HE + S) is independent of pH.[49] A similar argument applies to the set of intermediates ES_2, HES_2, and H_2ES_2. As a result, the ratios R_1 and R_2 are independent of pH, even though the actual rates involved may vary by large factors. Thus, according to eq 2, the observed isotope effect will not vary with pH unless the isotope effect on the bond-breaking step ($k_3{}^{14}/k_3{}^{15}$) varies with pH.[50] However, it is not likely that this isotope effect varies with pH for the following reason. The steady-state rate of hydrolysis of *N*-acetyl-L-tryptophanamide is invariant[21] from pH 8.0 to 9.4, but the nitrogen isotope effect changes from 1.010 to 1.006 in the same interval. Since R_1 and R_2 do not vary with pH, it follows that the isotope effect on the bond-breaking step varies under conditions where the rate of the step does not vary. Although not impossible, such a phenomenon seems unlikely, particularly in view of the large change in the magnitude of the isotope effect.

We are thus left with the conclusion that the variation of the nitrogen isotope effect with pH cannot be explained by the mechanism of eq 3. Some new mechanism is needed. Such a mechanism is eq 4. Equation



4 is written only to rationalize the observed isotope effects, and the intermediates in eq 4 are not identical with those discussed in connection with eq 1. The requirements concerning relative rates of various reactions of the intermediates in eq 4 (see below) are such that these intermediates must be distinct from those discussed previously. We will later derive a more general equation which rationalizes both the nitrogen isotope effects and all other available mechanistic data.

The equation for the observed isotope effect for the mechanism of eq 4 is eq 5.[51] The ratio R_3 is no longer

$$\frac{k^{14}}{k^{15}} = \frac{k_4{}^{14}/k_4{}^{15} + R_3}{1 + R_3} \qquad (5)$$

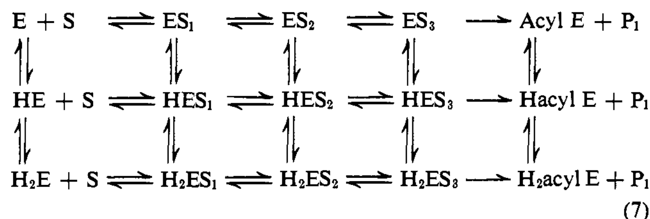
simply the ratio of two rate constants, but instead represents the partitioning of all forms of the ES intermediate between going on to acyl enzyme and returning to starting materials. The correction expression for R_3 in this case[52] is eq 6. If R_3 is not too different from

$$R_3 = \frac{k_4[\mathrm{ES}] + k_5[\mathrm{HES}] + k_6[\mathrm{H_2ES}]}{k_{-1}[\mathrm{ES}] + k_{-2}[\mathrm{HES}] + k_{-3}[\mathrm{H_2ES}]} \qquad (6)$$

unity, a nitrogen isotope effect will be observed and the isotope effect will vary with pH because of the variation of R_3 with pH. That is, whatever the intermediate in eq 4 is, its fate is pH dependent. The pH dependence of the observed isotope effect indicates that the intermediate has a greater tendency to return to starting material at pH 8 than at pH 6.7 or 9.4. It is not possible to assign this pH variation to individual rate constants in the reaction sequence. It is possible that the isotope effect on the bond-breaking step in eq 4 might also be pH dependent, but such dependence is not required in order to explain the observed pH variation of the isotope effect.

The new intermediate which we are proposing has not been identified previously in chymotrypsin-catalyzed reactions, but it might be identical with the tetrahedral intermediate which was proposed by Caplow.[32] Such a tetrahedral intermediate would fulfill our mechanistic criteria well, but we are unable to present a convincing argument for this identification of the intermediate.

The Final Mechanism. We must now reconsider the earlier kinetic data of Hess, *et al.*,[31] in light of the conclusions presented in the previous section and derive a kinetic mechanism which fits all of the available data. This mechanism is given in eq 7. The first two inter-



mediates are derived from the studies of Hess, *et al.*;[31] the first is presumably the Michaelis complex; the second may be the result of some conformational change of the enzyme. The third intermediate is the new one whose existence is implied by our isotope effect data. The ratio R for the fate of this intermediate must be near unity and must vary with pH. Decomposition of this intermediate results in formation of the acyl enzyme.

We are unable to specify whether intermediates ES_1 and H_2ES_1 are themselves reactive or whether they can react only after conversion to HES_1. It is possible that some of the rate constants in the scheme of eq 7 might be effectively zero. The principal contributions of our isotope effect study to this problem are the indication of the existence of intermediate ES_3 and the existence of parallel reaction paths for the acylation of the enzyme.

With the information at hand it should be possible to estimate magnitudes for at least some of the rate constants given in eq 7. However, the amount of data involved is too small to provide a convincing fit to the postulated mechanism.

(49) It is implicit in this treatment that proton transfers are much faster than other steps in the mechanism.

(50) If more than one step in eq 3 had a substantial isotope effect, the observed isotope effect would vary with pH if the isotope effect on any individual step varied with pH.

(51) We have assumed in eq 5 that the nitrogen isotope effect on the bond-breaking step in eq 4 is independent of the number of protons involved; that is, $k_4{}^{14}/k_4{}^{15} = k_5{}^{14}/k_5{}^{15} = k_6{}^{14}/k_6{}^{15}$. Although this assumption may not be precisely correct, the same substrate is involved in all cases, and it is unlikely that this assumption is seriously in error.

(52) For simplicity, all terms on the right side of eq 6 have been multiplied by ([ES] + [HES] + [H_2ES]). The equation for the pH dependence of R_3 appears complex, but involves only the ionization constants K_1 and K_2 and the rate constants k_4, k_5, k_6, k_{-1}, k_{-2}, and k_{-3}. The concentrations of the various intermediate species drop out.

The above discussion would have to be modified somewhat if more than one step in the reaction mechanism were subject to an appreciable nitrogen isotope effect. It is possible, for example, that if ES_3 in eq 7 were the tetrahedral intermediate, there might be an isotope effect on the formation of this intermediate, as well as on its decomposition. Although we cannot convincingly eliminate this possibility at this time, our feeling is that such an isotope effect would be small. If this proves not to be the case, a more complex treatment of these isotope effects will be necessary.

Finally, we should note that we have not taken account of the existence of various conformational forms of chymotrypsin which have been demonstrated by Lumry and others.[23-26] Such considerations add still further complexity to the kinetic mechanism of this well-understood enzyme.

Experimental Section

Measurements of pH were made with a Radiometer Model 26 pH meter at 22°. Standard buffers (E. H. Sargent and Co.) were used to calibrate the meter by the two-buffer method. Spectrophotometric measurements were made with a Gilford Model 222 photometer attached to a Beckman DU monochromater at 25.0°.

Materials. *N*-Acetyl-L-tryptophanamide was purchased from Sigma or synthesized as described by Huang and Niemann.[53] The product was recrystallized from methanol–pentane before use and had the expected melting point and spectral properties. The product was free of ammonia (*vide infra*). Deionized doubly distilled water was used for all solutions. The enzyme was desalted on Sephadex G-25 before use. Bovine α-chymotrypsin, lot 50C-2550, was obtained from Sigma. Magnesium oxide was Matheson Coleman and Bell reagent grade. Buffers were made from Baker KH_2PO_4, 0.05 *M*, adjusted to the proper pH with KOH.

Analyses. Traces of ammonia in aqueous solution were analyzed by the Nessler method.[54] Kinetic measurements of the chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophanamide were made spectrophotometrically at 306 nm, essentially as described by Bender, *et al.*[20] Approximately the same slit width was used in all measurements, and the extinction coefficient change resulting from the hydrolysis was found to be 70 M^{-1} cm^{-1}.

Isotopic analyses were conducted on a Nuclide Associates RMS 6-60 isotope-ratio mass spectrometer equipped with a double inlet system. Isotope ratios *m/e* 29/28 were measured alternately for the sample and for the tank standard, at least six such cycles being used for the calculation of the isotope ratio. All measurements were made at the same pressure. The lack of a peak at *m/e* 32 was used to confirm the lack of air in the samples.

Chymotrypsin Isotope Effects. A 20-ml portion and a 75-ml portion of *N*-acetyl-L-tryptophanamide (0.01 *M*) in phosphate buffer were equilibrated at 25.0° for at least 30 min. Measured portions of a freshly desalted chymotrypsin solution in the same buffer were then added and a 3-ml aliquot was withdrawn from the larger solution for spectrophotometric monitoring. The time required to reach approximately 10% reaction (usually about 30 min) was determined by monitoring the 3-ml aliquot at 306 nm and at the end of that time the reaction in the larger flask was stopped by addition of 0.85 g of Norit. The smaller sample, which contained a sixfold higher concentration of enzyme, was allowed to react for several hours in order to ensure that hydrolysis of the substrate was complete, and then 0.45 g of Norit was added. Each solution was filtered (Whatman no. 42 filter paper), treated with a second like portion of Norit, and filtered again. This procedure was demonstrated to remove more than 99.9% of the substrate. Each solution was then subjected to ultrafiltration in a Dia-Flo filtration apparatus with a UM-2 filter. The filtrate was then steam distilled in an all-glass apparatus similar to that described by Bremner.[55] For the experiments at pH 6.7, 0.1 g of MgO was added before steam distillation. The distillate was trapped with 5 ml of 0.1 *M* H_2SO_4. The progress of the distillation was occasionally monitored by Nessler analysis to ensure that all of the ammonia had been distilled before the distillation was stopped. The acidic distillate was concentrated to about 3 ml over a bunsen burner.

For oxidation and isotopic analysis the concentrated distillate was put in one arm of a Y tube and a like volume of NaOBr solution, prepared according to Bremner,[55] was put in the other arm. The solutions in the Y tubes were frozen in Dry Ice, evacuated to 10^{-4} mm pressure, thawed, refrozen, reevacuated, thawed, refrozen, and reevacuated. After the tube was warmed to room temperature the contents of the two sides of the Y tube were mixed and allowed to stand at room temperature for at least 30 min. The tube was frozen with liquid nitrogen and the evolved N_2 was pumped *via* an all-glass Toepler pump through a second liquid nitrogen trap and into a breakseal tube, which was quickly sealed with a torch.

Control Experiments. Elaborate precautions were taken to ensure that the only source of ammonia in the chymotrypsin experiments was the hydrolysis of the substrate. Nessler analysis of buffer samples which had been acidified and concentrated 25-fold showed no appreciable ammonia. When either chymotrypsin alone or substrate alone was carried through the Norit, filtration, steam distillation, and concentration steps, no ammonia could be detected.

Although the hypobromite oxidation procedure for nitrogen analysis has been used extensively, caution has been advised in its use for analyses where high precision is required. Repeated analysis of standard samples of ammonia which had been carried through our procedure all the way from the Norit step established that the entire procedure was reliable to about ± 0.000002 in the isotope ratio for a wide range of ammonia concentrations. Bremner has reported similar success.[55]

Nitrogen Exchange. A sample of *N*-acetyl-L-tryptophanamide was hydrolyzed with chymotrypsin at pH 9.43 in the presence of 0.002 *M* $^{15}NH_4Cl$ (8.6 atom % ^{15}N). The reaction was stopped after 50% completion by addition of H_2SO_4. The remaining substrate was isolated by extraction with ether and hydrolyzed with NaOH. The NH_3 thus produced was steam distilled and analyzed by the usual procedure. The resulting N_2 had an isotope ratio of 0.00947, which is not significantly higher than that of samples hydrolyzed in the absence of enriched NH_3.

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